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p14^{ARF} interacts with N-Myc and inhibits its transcriptional activity

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Abstract In this study, we report that the human p14^{ARF} associates in vivo with the N-Myc and inhibits N-Myc mediated transcriptional activation. We have determined that the region (aa 140–300) encompassing the N-Myc BoxIII is required for efficient interaction in vivo. Furthermore, we demonstrate that in the SK-N-BE neuroblastoma cell line p14^{ARF} over-expression delocalized N-Myc from the nucleoplasm into nucleoli and that N-Myc regions required for interaction with p14^{ARF} are also important for nucleoli co-localization. Finally, we determine that the N-terminal region of the p14^{ARF} protein is involved in binding to c-Myc and N-Myc proteins.

Keywords: p14^{ARF}; N-Myc; C-Myc; Protein interactions; Transcription; Tumor suppressor

1. Introduction

Genes of the Myc family contribute to the genesis of several types of human tumors. In all cases, Myc expression is increased in the tumors indicating that Myc elevated expression contributes to tumorigenesis [1,2]. N-Myc and c-Myc have different patterns of gene expression; whereas c-Myc is expressed during embryonic development and in adult tissues, N-Myc is found in undifferentiated types of cells in the lung, heart, nervous system, kidney, limbs, etc. [3,4].

N-Myc oncogene is implicated in the pathogenesis of neural crest derived tumors including neuroblastoma, the most frequent solid malignancy in infants. Amplification of N-Myc gene is the major negative prognostic marker in human neuroblastomas [5,6]. Although N-Myc and c-Myc share large regions of sequence conservation, their physiological role in the cell does not seem redundant since gene inactivation studies have shown that functional loss of either c-Myc or N-Myc leads to embryonic lethality [4,7–9].

Myc proteins act as transcription factors, although c-Myc transcriptional activity has been well characterized and several co-activators have been found, much less is known about N-Myc co-factors mainly because of the assumption that c-Myc and N-Myc have redundant functions [1,10].

Among c-Myc partners, the tumor suppressor protein ARF (alternative reading frame) has been found to directly bind the Myc protein and dramatically block c-Myc's ability to activate transcription and induce hyperproliferation and transformation [11–13].

Although ARF is not detectably expressed in most normal tissues, increased levels of mitogenic signals or aberrant expression of oncogenes induce the transcription of ARF. In particular, c-Myc was the first oncogene to be found to activate ARF that in turns, in order to induce cell cycle arrest, must functionally inhibit cell cycle regulated gene expression [14,15]. The p14^{ARF} protein has both p53-dependent and p53-independent tumor-suppressive activities. In a p53 background, ARF antagonizing the E3 ubiquitin ligase activity of MDM2 determines the stabilization of p53 incrementing its transcriptional activity; the result is the induction of cell-cycle arrest or apoptosis [15]. More recently, several ARF p53 independent functions have been documented. In particular, ARF has been shown to associate with transcription factors as Foxm1b, E2F, DP1, c-Myc, all of them directly involved in the regulation of cell cycle genes expression [11,15–17].

Given the similarity between N-Myc and c-Myc proteins but also the striking divergence among their expression pattern, we were interested to determine whether p14^{ARF} interacts and regulates N-Myc. We found that p14^{ARF} binds N-Myc in vivo and inhibits N-Myc transcriptional activity. We also found that ARF over-expression in neuroblastoma cells delocalizes the N-Myc protein from the nucleoplasm to the nucleoli where it co-localizes with ARF. We have mapped the N-Myc region involved in ARF binding in the central region of the protein (aa 140–300) and we have demonstrated that N-Myc mutants that do not interact with p14^{ARF} are not recruited into nucleoli upon ARF co-expression. Finally, we mapped in the N-terminal region of the p14^{ARF} protein the domain involved in c-Myc and N-Myc binding, respectively.

2. Materials and methods

2.1. Plasmids

pcDNA3-N-Myc, N-Myc d(1–300), N-Myc d(1–134), N-Myc d(20–90), and N-Myc d(96–140), N-Myc d(350–464), N-Myc d(350–464) were kindly provided by Dr. T. Fotsis [18]. pCMV14-N-Myc and GFP-p14^{ARF} have been previously described [19,13]. F:p14^{ARF}, F:p14^{ARF}(1–65), and F:p14^{ARF}(65–132) were obtained by subcloning the corresponding cDNA ARF coding sequences into the p3XFLAG-CMV10 vector (Sigma).

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2.2. Cell culture, luciferase assay and immunofluorescence

Human 293 T, SK-N-BE and Tet-21/N cell lines were grown in DMEM supplemented with 10% fetal calf serum. Tet-21/N cells [19] were maintained in the presence of tetracycline (1 μg/ml) for at least one week before transfection. 1×10^5 cells/well were plated and grown for an additional 24 h in 6 well plates in the presence of tetracycline. The cells were washed with 1× PBS and then transfected with the appropriate constructs by the polyethylenimine (PEI 25 K) method as described [20]. After transfections, the cells were incubated for 48 h in presence or absence of tetracycline as required. The activity of firefly was measured with the dual luciferase assay kit (Promega) according to the manufacturer's instructions by using a T20/20 luminometer (Turner Design). pRL-CMV (Promega) was co-transfected for normalization. The amounts of transfected plasmids DNAs are indicated in the legend to figures. For immunofluorescence analysis, SK-N-BE cells were transfected with lipofectamine with 600 ng each of GFP-p14^{ARF} and/or pcDNA3-N-Myc or pcDNA3-N-Myc d(1–300) plasmids and the cells were processed for immunofluorescence as described [13] using the N-Myc (C2, Santa Cruz Biotechnology) antibody.

2.3. Antibodies and co-immunoprecipitations

The following antibodies were used for the immunological techniques: anti-Myc (N262 for IP and 9E10 for WB, Santa Cruz Biotechnology, Inc.), anti-Max (C17, Santa Cruz Biotechnology, Inc.), anti-ARF (C-18, Santa Cruz Biotechnology, Inc.), and anti-N-Myc (C2, Santa Cruz Biotechnology). Co-immunoprecipitations from transiently transfected cells were carried out as previously described [13]. All interactions were carried out overnight at 4 °C. The beads were washed at least five times using buffer F (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 30 mM $Na_4O_7P_2$, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton and 0.1 mM PMSF) before loading on SDS–PAGE.

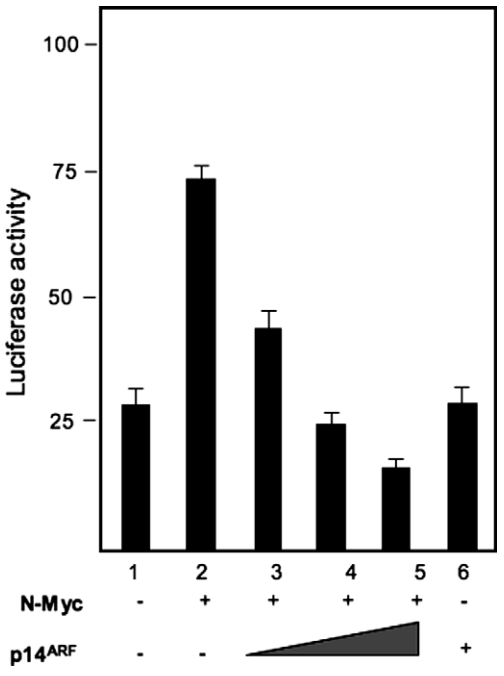

Fig. 1. ARF expression inhibits N-Myc activated transcription. Tet21Ncells were cotransfected with 100 ng of hTERT-Luc and different amounts (0.1; 0.5 and 1 μg, respectively) of F:p14^{ARF}, as indicated. N-Myc was activated by tetracycline withdrawal. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%.

3. Results and discussion

3.1. p14^{ARF} over-expression inhibits N-Myc transcriptional activity

It has been recently shown that ARF could inhibit c-Myc's ability to activate transcription [11–13]. We sought to determine whether p14^{ARF} might affect the transcription activity of N-Myc. To this end, we used a well characterized inducible cell line Tet-21/N, a human neuroblastoma cell line in which N-Myc expression is controlled through a Tet-off inducible promoter [19]. Tet-21/N cells were co-transfected with the hTERT-Luc construct and an expression vector for p14^{ARF}. N-Myc induction was obtained by tetracycline withdrawal and the cell extracts were analyzed for the human Telomerase promoter (hTERT) driven luciferase expression. As reported in Fig. 1, p14^{ARF} inhibits in a dose-dependent manner the N-Myc mediated activation of the human TERT promoter [21], while no effect was observed on basal expression of the hTERT promoter. Moreover, co-transfection experiments performed in the p53 null H1299 cell line gave similar results indicating that the decrease of N-Myc mediated transcription by ARF does not involve p53 (data not shown).

3.2. N-Myc associates with p14^{ARF} in vivo

We have previously demonstrated that p14^{ARF} directly associates with the c-Myc protein. Even if it is a general assumption that the interaction partners of c-Myc are also N-Myc partners, the failure of expression of either of the two Myc family members is not redundant and it cannot be excluded that they can form different complexes with their interactors.

To investigate the physical interaction between p14^{ARF} and N-Myc, Co-immunoprecipitation assays were performed with protein extracts from 293T transiently transfected cells. The cells were co-transfected with pcDNA3-N-Myc along with F:p14^{ARF} (F:ARF) and nuclear extracts were prepared and subjected to CoIP as reported in Fig. 2. IPs were carried out using either the N-Myc or the ARF antibodies, and the immunoprecipitated materials were analyzed by Western blotting with anti-N-Myc and anti-FLAG antibodies, respectively. The results reported in Fig. 2A showed that p14^{ARF} co-immunoprecipitated with N-Myc. In the converse experiment (Fig. 2B), using the ARF antibody for immunoprecipitation, N-Myc co-precipitated with ARF, whereas control IgG did not.

To identify N-Myc sequences involved in N-Myc-ARF interaction, we carried Co-IP analysis using protein extracts from 293T cells transiently co-transfected with F:ARF expression vector and several N-Myc deletion mutants as indicated in Fig. 3B. The protein extracts were immunoprecipitated with the ARF antibody and the Co-IP proteins analyzed by WB with N-Myc and FLAG antibodies, respectively. As shown in Fig. 3A, the N-Myc deletion mutant d(1–300) did not associate with p14^{ARF} (lane 10), suggesting a crucial role of the N-terminus portion of the protein in ARF-binding. However, deletions of either BoxI d(20–90) or BoxII d(96–140) retained ARF-binding. The strength of interaction of the d(20–90) and d(1–134) mutants was reproducibly higher than that of the other N-Myc deletions; we do not have an explanation for this behavior but we can speculate that these deletions could better expose the N-Myc domain for ARF binding. From these data we conclude that the N-Myc region involved in p14^{ARF} interaction resides in the region from aa 140 to 300.

3.3. p14^{ARF} induces N-Myc nucleolar co-localization

It has been shown that c-Myc and ARF co-localize in the same cellular compartment upon ARF over-expression (11–

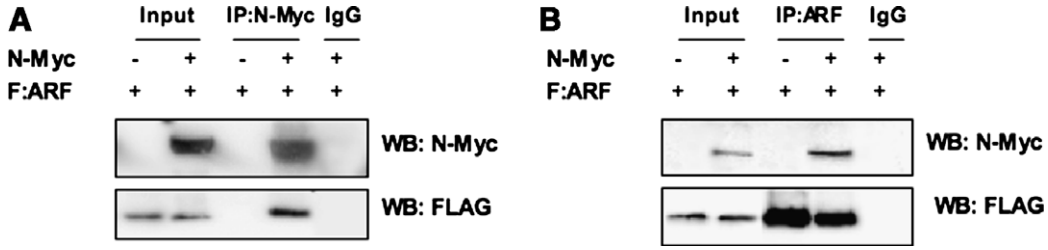

Fig. 2. p14^{ARF} interacts with N-Myc in vivo. 293T cells were transfected with F:ARF in the presence or absence of pcDNA3-N-Myc, as indicated. (A) Protein extracts were IP with the N-Myc antibody or preimmune antiserum (IgG) as negative control, and the co-purified complexes were analyzed by WB with N-Myc and FLAG antibodies, as indicated. (B) The same protein extracts utilized in panel A were IP with the ARF antibody and the Co-IP complexes were analyzed by WB with N-Myc and FLAG antibodies. Five percent of protein inputs were loaded as shown.

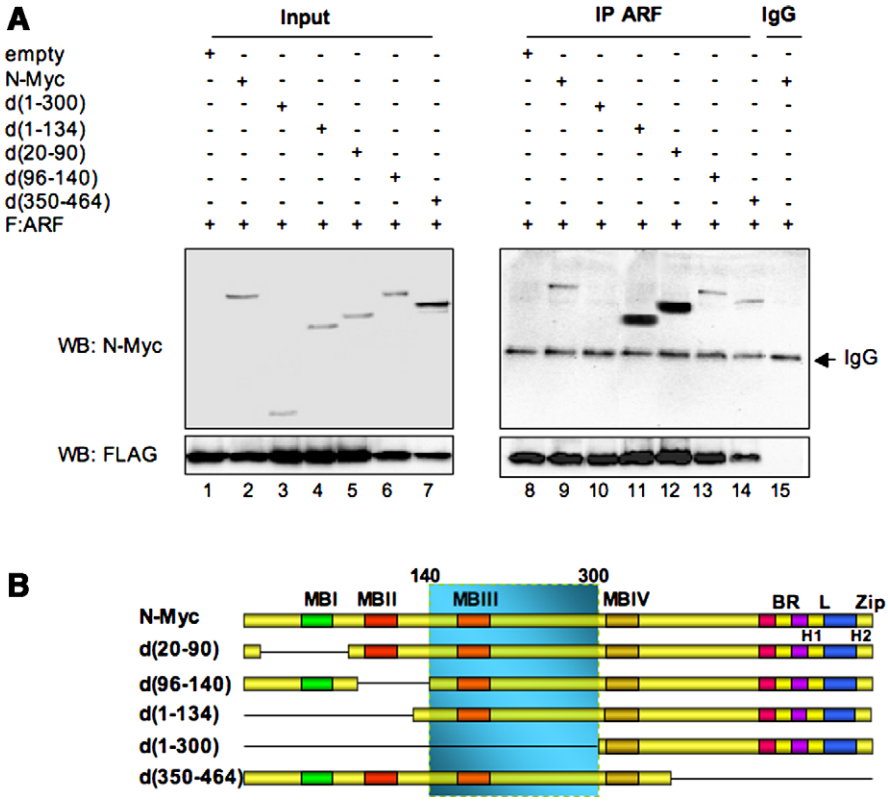

Fig. 3. Mapping the N-Myc domain involved in ARF binding. (A) 293T cells were co-transfected with F:ARF and a series of N-Myc deletion constructs as indicated. Protein extracts were IP with the ARF antibody or with preimmune antiserum (IgG) as negative control. (B) Schematic representation of the N-Myc full-length protein and deletion mutants. The relative strengths of interactions with p14^{ARF} are indicated.

13). To investigate whether N-Myc co-localizes with ARF in the same cellular compartment, SK-N-BE cells were transfected with the N-Myc expression vector alone or in combination with the GFP-p14^{ARF}. In cells transfected with N-Myc the protein was found exclusively in the nuclear compartment while the GFP-p14^{ARF} showed a predominantly nucleolar localization (Fig. 4, panel A). In cells co-transfected with the two expression vectors, the GFP-p14^{ARF} protein retains the nucleolar localization while the N-Myc protein was found in the nucleolar compartment in 78% of the co-transfected cells (Fig. 4, panel B). We then tested the ability of the N-Myc d(1–300) protein, unable to interact with ARF (Fig. 3), to be recruited by ARF.

As shown in Fig. 4B, in the cells co-transected with both GFP-p14^{ARF} and N-Myc d(1–300) deletion mutants, the two proteins were found in the nucleoli and nucleoplasm, respectively. These data strengthen our findings that p14^{ARF} interacts in vivo with N-Myc, and a deletion of the central region of N-Myc protein strongly impairs interaction with ARF as well as sub-cellular co-localization in the nucleoli.

3.4. *p14^{ARF} N-terminal domain is involved in interaction with Myc proteins*

In the attempt to define the ARF region involved in Myc interaction, we subcloned the ARF cDNA regions coding for aa 1–65 and for aa 65–132 in a FLAG epitope tagged CMV10 vector. The F:ARF(1–65), F:ARF(65–132) constructs and the wt F:ARF vectors were transfected alone or in combination with N-Myc and c-Myc vectors into 293T cells as indicated in Fig. 5A and B. Protein extracts from the transfected

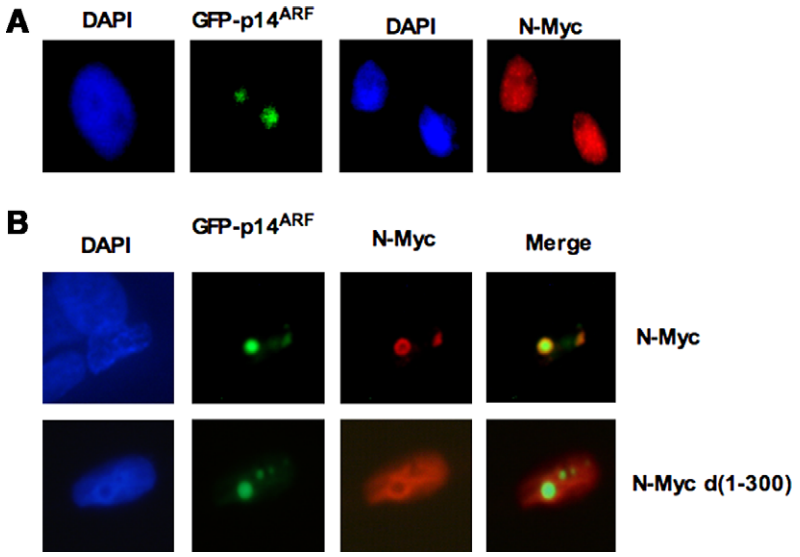

Fig. 4. $p14^{ARF}$ overexpression recruits N-Myc into nucleoli. SK-N-BE cells were co-transfected with GFP-$p14^{ARF}$ and pcDNA3-N-Myc or pcDNA3-N-Myc d(1–300) by Lipofectamine, as indicated. An example of immunofluorescence microscopy of the cells immunostained with N-Myc and analyzed by fluorescence microscopy as previously described is shown. At least 150 cells were analyzed in each experiment. Values are means from three independent experiments. All images were digitally processed using Adobe Photoshop.

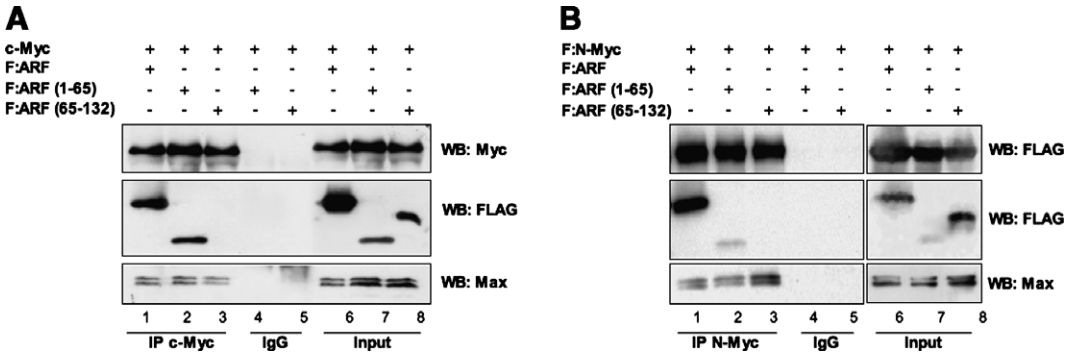

Fig. 5. (A) 293T cells were co-transfected with 7 μg each of pcDNA3-c-Myc together with the F:ARF construct or its deletion mutant derivatives F:ARF (1–65) and F:ARF (65–132). Protein extracts were immunoprecipitated with the c-Myc antibody (lanes 1–3) or a preimmune antiserum IgG (lanes 4 and 5) as negative control. Co-IP complexes were analyzed by WB with the indicated antibodies. Five percent of the protein inputs were loaded in lanes 6–8. (B) 293T cells were co-transfected with F:N-Myc together with the F:ARF construct or its deletion mutant derivatives F:ARF(1–65) and F:ARF(65–132). Protein extracts were immunoprecipitated with the N-Myc antibody (lanes 1–3) or IgG (lanes 4 and 5) as negative control, as indicated. Co-IP complexes were analyzed by WB with the indicated antibodies. Five percent of the protein inputs were loaded in lanes 6–8.

cells were immunoprecipitated either with the c-Myc (panel A) or the N-Myc (panel B) antibodies and the coimmunoprecipitated proteins analyzed by WB with c-Myc, N-Myc, Max and FLAG antibodies as indicated.

The results shown in Figs. 5A and B demonstrate that either the ARFwt protein or the protein encoding for the first 65 aa interacts with c-Myc and N-Myc, respectively. In contrast, the ARF C-terminal domain from aa 65 to 132 is impaired in binding to either Myc proteins. As control of c-Myc and N-Myc immunoprecipitation, WB with the Max antibody confirmed the presence of the endogenous Max protein in all the Myc IP extracts, suggesting that $p14^{ARF}$ associates with N-Myc-Max heterodimer in vivo and that N-Myc binding to Max and $p14^{ARF}$ is not mutually exclusive.

3.5. Conclusion

Taken together the data presented in this study demonstrated that $p14^{ARF}$ is a bona fide N-Myc partner, and that N-Myc mediated transactivation is inhibited by ARF over-expression. In addition, ARF over-expression in the neuroblastoma SK-N-BE cell line recruits N-Myc into nucleoli. Mapping of the N-Myc domain involved in ARF binding reveals that a region between aa 140 and 300 containing the Myc BoxIII is crucial for N-Myc-ARF binding. Moreover, the same region is also required for nucleolar co-localization. These findings underlie the relevance of the Myc BoxIII domain in the physiological interaction between the ARF and N-Myc proteins. Finally, we demonstrated that the N-terminal region of the ARF protein (aa 1–65) is necessary for ARF binding to both c-Myc and N-Myc proteins.

Myc proteins have been dissected in several biologically functional domains and an enormous number of studies have been done trying to give valuable insight into their contribution to Myc functions [22]. Myc BoxII has been found to be essential for most Myc functions such as transformation, apoptosis differentiation and cell cycle arrest. Deletion of this

box dramatically reduces the transactivation function of Myc [10,22]. However, it has also been found that deletion of MycBoxII reduces the transcription of most but not all Myc target genes [23]. Two further Myc homology domains have been characterized, MycBoxIII and MycBoxIV, and it has been found that deletion mutants in these regions have defects in the activation of a variable set of Myc target genes [10,24,25]. We have found that while the MycBoxII seems to be dispensable for N-Myc binding to ARF, efficient interaction between the two proteins requires a region encompassing the MycBoxIII. However, we cannot exclude the contributory role of additional N-terminal regions of N-Myc in ARF binding.

How ARF binding to Myc might inhibit its transactivating functions is unknown. Myc mediated transactivation is the result of a complex network of protein interactions between Myc proteins and their co-regulators and regulation of transcription by Myc is supposed to be the consequence of formation of dedicated Myc protein complexes. Recently, we have shown that ARF does not possess an intrinsic repression activity, and as discussed recently ([15] and references therein) it is likely that ARF binding may alter the association of Myc to dedicated co-factors either through sterical hindrance or due to mutually exclusive interactions.

In conclusion, the findings reported here showing that ARF negatively regulates N-Myc, in a manner similar to c-Myc, provide additional evidences of the p53-independent tumor-suppressor activity of the ARF protein.

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